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Fah1p, a *Saccharomyces cerevisiae* Cytochrome b_5 Fusion Protein, and Its *Arabidopsis thaliana* Homolog That Lacks the Cytochrome b_5 Domain Both Function in the α-Hydroxylation of Sphingolipid-associated Very Long Chain Fatty Acids*

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A search of the *Saccharomyces cerevisiae* genome data base for cytochrome b_5-like sequences identified a 1.152-kilobase pair open reading frame, located on chromosome XIII at locus YMR272C (*FAH1*). That gene encodes a putative 384-amino acid protein with an amino-terminal cytochrome b_5 domain. The b_5 core domain shows a 52% identity and 70% similarity to that of the yeast microsomal cytochrome b_5 and a 35% identity and 54% similarity to the b_5 core domain of *OLE1*, the *S. cerevisiae* Δ-9 fatty acid desaturase. Expression of the *S. cerevisiae FAH1* cytochrome b_5 domain in *Escherichia coli* produces a soluble protein that exhibits the typical oxidized *versus* reduced differential absorbance spectra of cytochrome b_5.

Sequence analysis of Fah1p reveals other similarities to Ole1p. Both proteins are predicted to have two hydrophobic domains, each capable of spanning the membrane twice, and both have the $HX_{(2-3)}(XH)H$ motifs that are characteristic of membrane-bound fatty acid desaturases. These similarities to Ole1p suggested that Fah1p played a role in the biosynthesis or modification of fatty acids.

Disruption of the *FAH1* gene in *S. cerevisiae* did not give any visible phenotype, and there was no observable difference in content or distribution of the most abundant long chain saturated and unsaturated 14–18-carbon fatty acid species. Northern blot analysis, however, showed that this gene is expressed at much lower levels (~150-fold) than the *OLE1* gene, suggesting that it might act on a smaller subset of fatty acids. Analysis of sphingolipid-derived very long chain fatty acids revealed an approximately 40-fold reduction of α-HO 26:0 and a complementary increase in 26:0 in the gene-disrupted *fah1*Δ strain. *GAL1* expression of the *S. cerevisiae FAH1* genes in the *fah1*Δ strain restores α-HO 26:0 fatty acids to wild type levels. Also identified are a number of homologs to this gene in other species. Expression of an *Arabidopsis thaliana FAH1* gene, which does not contain the cytochrome b_5 domain, in the *fah1*Δ strain produced an approximately 25-fold increase in α-HO 26:0 and reduced the levels of its 26-carbon precursor, suggesting that it functions in very long chain fatty acid hydroxylation using an alternate electron transfer mechanism.

Cytochrome b_5 is a ubiquitous eukaryotic membrane protein that is an essential component of a number of endoplasmic reticulum (ER)[1]-linked redox enzyme systems. One of its primary roles in animal cells is in the formation of unsaturated fatty acids. In the process of double bond formation, the membrane-bound cytochrome b_5 transfers electrons by lateral diffusion from NADH cytochrome b_5 reductase to the Δ-9 fatty acid desaturase (1, 2). Cytochrome b_5 also appears to function in other fatty acid-modifying reactions. At least one step in the formation of very long chain fatty acids (20–26 carbons) is thought to be cytochrome b_5-dependent (3). These membrane-bound fatty acid elongation systems act independent of the soluble fatty acid synthase complex (4). Their very long chain products, which in yeast are predominantly 26 carbons in length (26:0),[2] are minor but physiologically important fatty acids that are incorporated into sphingolipids. In these lipids, the hydrophobic ceramide portion is composed of the long chain base phytosphingosine, which is amide-linked to the very long chain fatty acid, the majority of which is hydroxylated at the α-position (5, 6). It is suggested that cytochrome b_5 is also the intermediate electron donor for α-hydroxylation (7) and that this reaction appears to involve the direct hydroxylation of a sphingolipid-bound fatty acid (8, 9). The oleate 12-hydroxylase (*FAH12*) activity of *Ricinus communis* is also shown to be dependent upon cytochrome b_5 (10).

The *Saccharomyces cerevisiae* genome contains a number of sequences related to cytochrome b_5. A gene encoding a microsomal cytochrome b_5 analogous to the mammalian enzyme, was cloned by Truan *et al.* (11). It is a small 120-amino acid polypeptide that contains the typical carboxyl-terminal hydrophobic membrane-anchoring sequence that is necessary for its binding to the ER (12). We also recently identified a cytochrome b_5-like sequence as a carboxyl-terminal extension to the Δ-9 fatty acid desaturase (*OLE1*). This gene appears to have originated from a fusion of the ancestral cytochrome b_5 and fatty acid desaturase genes. The cytochrome b_5 domain of *OLE1* was shown to be essential for desaturase activity (13).

The *S. cerevisiae* Ole1p is not the only occurrence of a cytochrome b_5 gene fusion in eukaryotes. Our analysis of the *Histoplasma capsulatum* Δ-9 fatty acid desaturase reported by Gargano *et al.* (14) indicates that it also possesses a cytochrome b_5 carboxyl-terminal extension. An amino-terminal cytochrome

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[1] The abbreviations used are: ER, endoplasmic reticulum; OLE1p, *S. cerevisiae* Δ-9 fatty acid desaturase protein; ORF, open reading frame; GC, gas chromatography; Fah1p, *S. cerevisiae* fatty acid hydroxylase protein; PCR, polymerase chain reaction; EST, expressed sequence tag.

[2] Fatty acids are denoted by a standard designation that indicates the number of carbons, followed by the number of double bonds (*e.g* 26:0, a 26-carbon fatty acid with no double bonds; 16:1, a 16-carbon fatty acid with one double bond).

b_5 fusion to a protein similar to an acyl lipid desaturase was found in *Arabidopsis* (15), and recently an amino-terminal cytochrome b_5 to a Δ-6 fatty acid desaturase was identified in *Borago officinalis* (16).

A search of the *S. cerevisiae* genome[3] revealed a presumptive cytochrome b_5-containing gene, YMR272C, which also had the characteristics of a fatty acid desaturase. These enzymes have been shown to contain the conserved general motif $HX_{(2-3)}(XH)H$, where the histidine residues act to coordinate a μ-oxo-bridged diiron cluster (Fe-O-Fe) that functions as part of the reaction center (18, 19). These motifs are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (*FAH12*) (10), and in the yeast and human methyl sterol oxidase (*Erg25*) (22). The YMR272C ORF contains five putative $HX_{(2-3)}(XH)H$ motifs that are conserved in its homologs.

On the basis that this gene might encode a fatty acid-modifying enzyme, GC fatty acid methyl ester profiles of wild type and YMR272C gene-disrupted strains were compared. While no changes in fatty acid desaturation were observed, a peak identified as α-HO 26:0 was shown to be reduced in the disruption strain.

MATERIALS AND METHODS

PCR Cloning, Sequencing, and Disruption of the YMR272C ORF—Standard molecular biological techniques were used for all cloning procedures (25, 26). Vent DNA polymerase (New England Biolabs) was used for the amplification reactions, and the resulting PCR products were subcloned into the pCRscript SK+ vector (Stratagene). Three independent amplifications were confirmed by Sequenase sequencing (U.S. Biochemical Corp.). The PCR primers AGM039 and AGM040 (Life Technologies, Inc.) (Table I) were designed to allow amplification of the YMR272C ORF (chromosome XIII base pair coordinates 810777–809623) from DTY-11A genomic DNA (Table II). This PCR product was subcloned to create pAM109. To prepare a gene disruption, the *Hin*dIII site of pAM109's multiple cloning site was first destroyed by Klenow fill in and religation (pAM111). This plasmid was then cut as *Bst*EII/*Nco*I, blunt-ended with Klenow, and religated with *Hin*dIII linkers (pAM113). A *Hin*dIII-linked *LEU2* gene was ligated into this site (pAM116). A linear *Not*I/*Pst*I DNA fragment was excised from pAM116 and used for transformation of the DTY-10A and 11A strains. The YEASTMAKER yeast transformation system (CLONTECH) was used for these transformations. The resulting transformants were confirmed by PCR and Northern blot analysis, as described previously (13).

PCR Cloning of an Arabidopsis thaliana Homolog—A search of the *Arabidopsis* EST data base revealed a number of overlapping sequences with homology to the *S. cerevisiae* YMR272C ORF (Z32613, R65395, T20869, T21629, Z30502, N38440, Z32612, Z25612, and Z32614). PCR primers AGM045 and AGM046 (Table I) were designed to allow amplification of this open reading frame (GenBank™ accession number AF021804) from an *A. thaliana* cDNA library (17). The resulting PCR product was subcloned to create pAM129.

GAL1 Expression—For overexpression of the YMR272C and *A. thaliana* ORFs in yeast, blunt *Sac*I/*Bam*HI fragments of pAM109 and pAM129 were independently ligated into a blunt *Hin*dIII/*Bam*HI-cut YCpGAL plasmid (containing the galactose-inducible (*GAL1*) promoter) to create pAM132 and pAM133, respectively. These plasmids were then transformed into the YMR272C gene disruption strain (*fah1* Δ) (Table II).

Expression of the Cytochrome b_5 Domain in Escherichia coli—The cytochrome b_5 domain was excised from pAM109 using *Bst*YI, which cuts within YMR272C and at the *Bam*HI site of the polylinker. This 471-base pair fragment was subcloned into the *Bam*HI site of pET11d (Novagen) and orientation establishing (pAM151). To remove the remaining polylinker and to restore the reading frame, pAM151 was cut as *Nco*I and *Sal*I, blunt ended with Klenow, and religated (pAM154). The pET11d and pAM154 plasmids were transformed into the BL21(DE3) pLysS *E. coli* expression strain. To induce expression of the recombinant protein, 2 ml of an overnight culture were used to inoculate 100 ml of LB (200 μg/ml ampicillin). The cultures were then grown with shaking (250 rpm) at 37 °C for 2 h. Thirty minutes before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside, a 75 μg/liter concentration of the heme precursor 5-aminolevulinic acid was added (27), and the cells were then grown for 20 h at the reduced temperature of 30 °C (28). The cells were harvested by centrifugation at 5000 × *g* for 5 min and washed once with 100 mM Tris-HCl, pH 8, 1 mM phenylmethylsulfonyl fluoride. Cells were then lysed in the same buffer by French press, and the extract was centrifuged at 30,000 × *g* for 30 min. The soluble fractions were analyzed using a Perkin-Elmer Lambda 12 spectrometer. Redox absorbance spectra (400–600 nm) were obtained by comparison of air-oxidized against sodium dithionite-reduced samples. Cytochrome b_5 content was determined from the α-band absorbance maxima (558 nm) of the reduced cytochrome b_5 using an absorption coefficient of 26.5 mM^{-1} cm^{-1} (29).

Fatty Acid Analysis and Sphingolipid Extraction—Fatty acid methyl esters were prepared by HCl methanolysis as described previously (24). Gas chromatography was performed on a Varian 3400CX GC using a Supelcowax®10 30 m × 0.32 mm column (Supelco) run at 240 °C. Data were collected and analyzed using the Class-VP Chromatography Data System version 4.1 (Shimadzu Scientific Instruments) software. Gas chromatographic electron-ionizing mass spectroscopy was performed on a Varian 3400 gas chromatograph connected to a Finnigan MAT 8230 mass spectrometer, using the same column under similar conditions. α-Hydroxyhexacosanoic acid (α-hydroxy 26:0) was obtained from Sigma.

Sphingolipid and glycerolipid fatty acids were fractionated from logarithmic phase cells as described by Pinto *et al.* (30). 5% trichloroacetic acid-washed cell pellets were subjected to mild alkaline hydrolysis. The methanolic-KOH extract, which contains saponifiable fatty acids, was then acidified and removed from the cell pellet by centrifugation. Sphingolipids were then extracted from the saponified cell pellets with an ethanol/water/diethylether/pyridine/NH_4OH (15:15:5:1:0.018) solvent. After drying under nitrogen, sphingolipid fatty acid methyl esters were prepared by HCl methanolysis.

RESULTS AND DISCUSSION

Identification of S. cerevisiae Cytochrome b_5-like Sequences—Cytochrome b_5 is an essential component of a number of ER-linked redox enzyme systems. It functions in the modification of xenobiotic substances by P450 enzymes (35) as well as in a number of fatty acid-modifying enzyme systems, such as desaturation (1, 2), elongation (3), and hydroxylation (10, 36). A number of cytochrome b_5 fusion proteins have recently been reported. These are the *OLE1* gene, where the cytochrome b_5 is a carboxyl-terminal extension to the desaturase domain, and two plant genes that include an *Arabidopsis* amino-terminal cytochrome b_5 fusion to a polypeptide similar to the plant acyl lipid desaturases (15) and a Δ-6 fatty acid desaturase identified in *B. officinalis* (16). The animal Δ-9 fatty acid desaturases do

TABLE I
PCR primers

PCR primer	Sequence
AGM039 (Forward)	ACTAGTACGAAGA*TG*TCGACTAATACTTCC
AGM040 (Reverse)	TTGACAATTTTGGACGAGGCTGACC
AGM045 (Forward)	TCCATCAGTAAAAGGTCTGCAAAA*TGG*
AGM046 (Reverse)	AGATTAAACATCTCAAAATTCAGAAGG

TABLE II
S. cerevisiae strains used in this study

Strain	Genotype	Source
DTY-10A	*MATα, CYTb$_5$, OLE1, FAH1, leu2–3, leu2–112, TRP1, can1–100, ura3–1, ade2–1, his3–11, his3–15*	This laboratory
DTY-11A	*MATα, CYTb$_5$, OLE1, FAH1, leu2–3, leu2–112, trp1–1, can1–100, ura3–1, ade2–1, HIS3*	This laboratory
*fah1*Δ	*MATα, CYTb$_5$, OLE1, fah1*Δ*::LEU2, trp1–1, can1–100, ura3–1, ade2–1, HIS3*	This laboratory

[3] Available on the World Wide Web at http://genome-www.stanford.edu/saccharomyces.

not possess this cytochrome b_5 extension and are dependent on the diffusible microsomal cytochrome b_5 for activity (13). Our previous observation that the rat desaturase can function in an *ole1*Δ strain is due to the existence of the diffusible microsomal cytochrome b_5 protein, which provides electrons to the mammalian enzyme. The b_5 fold is also found in a number of other heme-binding proteins such the yeast cytochrome b_2, sulfite oxidases, and nitrate reductases (37).

In the x-ray crystal structure of the bovine cytochrome b_5, residues 21–78 define a heme-containing crevice, the walls of which are formed by two roughly antiparallel α-helices, with a floor of β-pleated sheets (31). Histidines 36 and 63 of this region are responsible for binding the heme iron group. This region is highly conserved among eukaryotic cytochromes b_5 and is referred to as the cytochrome b_5 "fold." The homologous cytochrome b_5 fold amino acid sequence of the yeast microsomal protein (32) was used for the data base search. This search identified a number of cytochrome b_5-like sequences (Table III and Fig. 1), including the microsomal cytochrome b_5, the b_5 domain of *OLE1*, cytochrome b_2, and a previously undescribed sequence that has 52% identity and 70% similarity to the yeast microsomal cytochrome b_5 heme binding domain. This predicted 1.152-kilobase pair open reading frame is located on chromosome XIII at locus YMR272C and encodes a 384-amino acid protein that contains an approximately 100-amino acid amino-terminal cytochrome b_5 domain (Fig. 2).

A characteristic of the microsomal cytochrome b_5 is the abundance of acidic residues (Glu and Asp) located within the region that includes the heme binding pocket. These residues appear to be part of a highly dynamic surface that is able to adapt its conformation to interact with numerous substrates (33). Fig. 1 shows that this region of the microsomal cytochrome b_5 has 13 such residues. YMR272C is comparable in having 11, while Ole1p and cytochrome b_2 each have only 4 of these residues. It was previously proposed (13) that the lower number of charged residues in the b_5 domain of Ole1p results from the reduced need to form many charge-pair interactions with the desaturase domain because this domain is directly linked to its electron-accepting substrate. The closer homology of the b_5 domain of YMR272C to the native cytochrome b_5 may reflect a more recent evolutionary fusion event than the b_5 fusion to the Δ-9 fatty acid desaturase. The different positions of their b_5 domains also suggest that the these two fusions occurred as independent evolutionary events rather than the divergence of a common b_5 fused ancestral gene.

PCR Cloning and Disruption of the YMR272C Open Reading Frame—The YMR272C ORF was PCR-amplified from wild type DTY-11A genomic DNA, and this sequence was disrupted with the *LEU2* gene as described under "Materials and Methods." Linear DNA disruption of this gene in either DTY-10A or 11A did not give any visible phenotype. Wild type levels of growth were observed on media containing either glucose or the nonfermentable carbon source glycerol.

Present in the GenBank™ data base are a number of homologs to YMR272C (Z49260). Table IV shows these homologies, and Fig. 3 shows the peptide sequence alignment of these ORFs. Based on the presence of the conserved histidine resi-

TABLE III
Results of a search of the S. cerevisiae genome database for the microsomal cytochrome b_5 fold motif

Gene	Chromosome	Locus	Identity	Similarity
			%	%
Cytochrome b_5	XIV	YNL111C	100	100
Identified as *FAH1*	XIII	YMR272C	52.2	69.6
OLE1	VII	YGL055W	43.5	60.9
Cytochrome b_2	XIII	YML054C	35.3	60.0

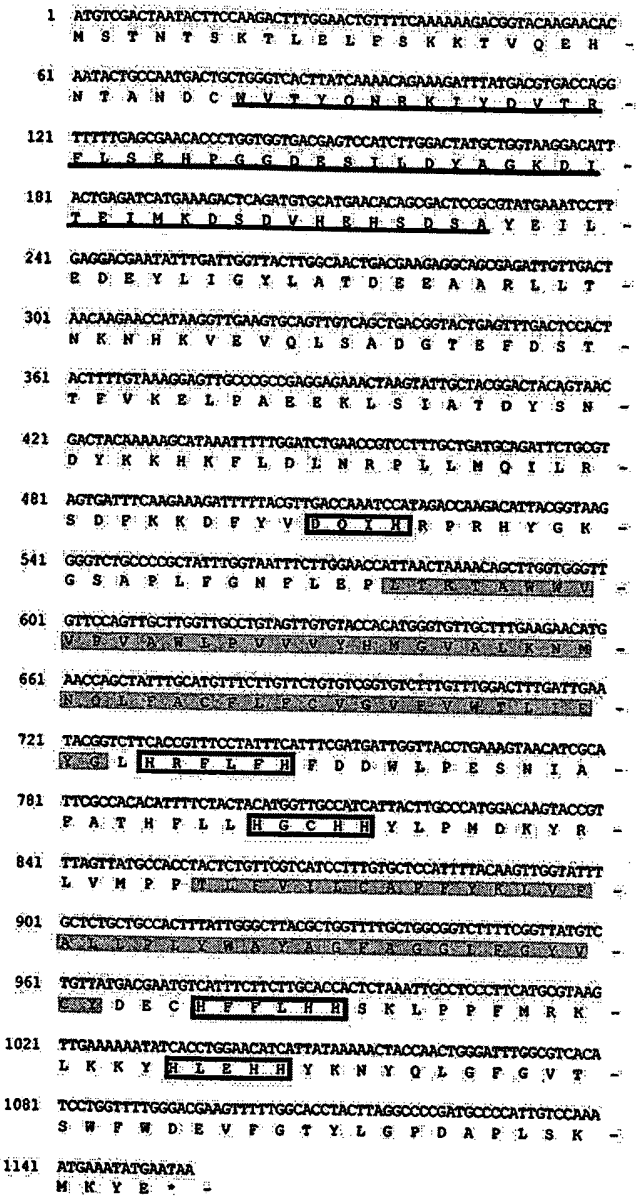


FIG. 2. **Nucleotide and amino acid sequence of the YMR272C ORF.** Marked are the cytochrome b_5 core domain (*underlined*), histidine motifs (*boxed*), and the transmembrane domains (*shaded*).

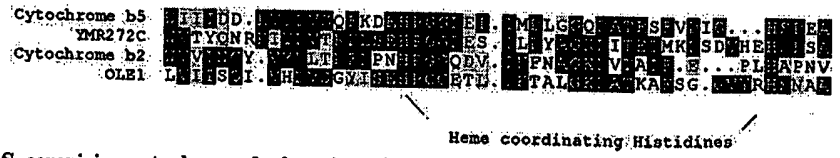


FIG. 1. **Alignment of *S. cerevisiae* cytochrome b_5 domains.** The cytochrome b_5-like domains identified in Table III were aligned using the GCG Pileup program. Homologies between these sequences were highlighted using the BOXSHADE program (available on the World Wide Web at http://ulrec3.unil.ch/software/BOX_form.html).

dues of the b_5 fold, the *Caenorhabditis elegans* (Z81038) sequence appears to have a cytochrome b_5-like region; however, the *Schizosaccharomyces pombe* (Z97209) and a reconstructed *A. thaliana* sequence (Fig. 4) do not.

E. coli Expression of the YMR272C Cytochrome b_5 Domain—To demonstrate that the N-terminal extension of YMR272C coded for a cytochrome b_5 domain, a 471-base pair fragment was cut from the pAM109 plasmid, and subcloned into the pET11d *E. coli* expression vector, as described under "Materials and Methods." This fragment encodes 150 amino acids of a soluble cytochrome b_5. As reported for the expression of other cytochromes b_5 (16, 34), after 20 h of induction at the lower temperature of 30 °C (28), cells containing the cytochrome b_5 sequence appear reddish in color, as compared with pET11d control cell inductions. Spectral analysis of a soluble extract from these cells demonstrates the oxidized *versus* reduced absorbance differential typical of a cytochrome b_5. Absorbance maxima are observed at 558 and 426 nm (Fig. 5). Using the absorption coefficient of 26.5 mM^{-1} cm^{-1} for the α-band (558 nm) (29), the cytochrome b_5 content was determined to be 7.6 μg/mg of soluble protein extract. This low expression level compares with that reported by Sperling *et al.* (15) for the expression of the *Arabidopsis* acyl desaturase cytochrome b_5 domain. Similarly, the recombinant protein was not detected by SDS-PAGE in initial soluble cell lysate (data not shown).

PCR Cloning of the A. thaliana FAH1 Open Reading Frame—A number of *A. thaliana* ESTs that have homology to the *S. cerevisiae FAH1* gene are present in the GenBank™ data base. This sequence information, which covers almost the whole ORF, allowed us to design PCR primers to amplify the entire coding sequence from a cDNA library (as described under "Materials and Methods"). The sequence of the resulting PCR was found to be consistent with the overlapping EST data, except for a single base discrepancy (C to A at base pair 565; Fig. 4), which converts a threonine to a lysine residue. The lysine residue appears to be more consistent with the multiple sequence alignment data shown in Fig. 3. There is a region of this ORF that is not covered by an EST. The sequence and ORF presented for this region (*lowercase letters* in Fig. 4) comes from sequenced PCR products (GenBank™, accession number AF021804). This *A. thaliana* Fah1p shows 42.6% identity and 62.1% similarity to the *S. cerevisiae* Fah1p. From the EST data there does not appear to be any cytochrome b_5-like sequences in any reading frame 5′ to the predicted start methionine.

Sequence Analysis ($HX_{(2-3)}(XH)H$ Motifs)—Examination of the non-b_5 domain of YMR272C revealed a number of motifs that are found in lipogenic enzymes. Sequence comparisons between known membrane-bound desaturases, including *OLE1* and the *Arabidopsis* sequence (15), revealed the conserved general motif $HX_{(2-3)}(XH)H$. These are a characteristic of membrane-bound desaturases (19) but are also found in the bacterial alkane hydroxylase (20) and xylene monooxygenase (21), in the plant oleate 12-hydroxylase (10), and in the yeast and human methyl sterol oxidase (*Erg25P*) (22). The histidine residues act to coordinate a μ-oxo diiron cluster (Fe-O-Fe) that functions as part of the reaction center. Removal of any one of these histidines from the rat Δ-9 fatty acid desaturase was shown to disrupt desaturase function (19). Ole1p contains three of these motifs, two HX_2HH and one HX_3H. Examination of the *S. cerevisiae*, *S. pombe*, *C. elegans*, and *A. thaliana* amino acid sequences shows five conserved histidine-rich motifs (two HX_2HH, one HX_3HH, a HX_4H, and a $(D/H)X_2H$). Figs. 2–4 show the sequence and positioning of these motifs.

Hydrophobic Analysis—Stukey *et al.* (38) proposed a model for Ole1p in which there are two long hydrophobic domains, each capable of spanning the membrane twice (Fig. 6). This allows the three histidine-containing motifs to lie on the cytoplasmic face of the ER membrane. Similarly, transmembrane

TABLE IV

Organism	Accession number	Identity	Similarity
		%	%
S. cerevisiae	Z49260	100	100
S. pombe	Z97209	46.0	67.7
A. thaliana	[a]	42.6	62.1
C. elegans	Z81038	36.1	59.9

[a] See "Materials and Methods."

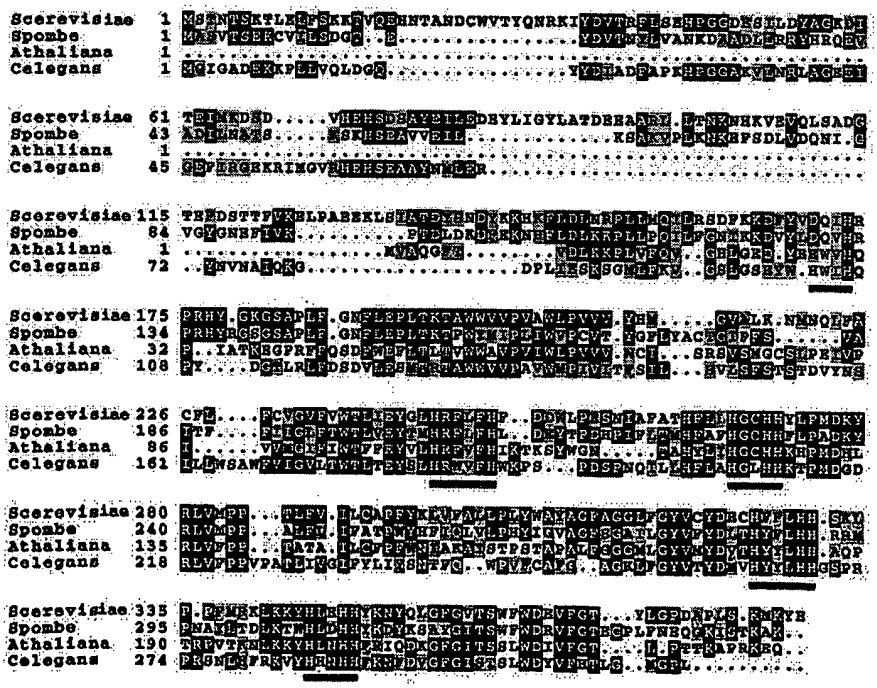


FIG. 3. **Alignment of the *FAH1* homologs.** The homologs of *FAH1* were aligned using the GCG Pileup program. Homologies between these sequences were highlighted using the BOXSHADE program (available on the World Wide Web at http://ulrec3.unil.ch/software/BOX_form.html).

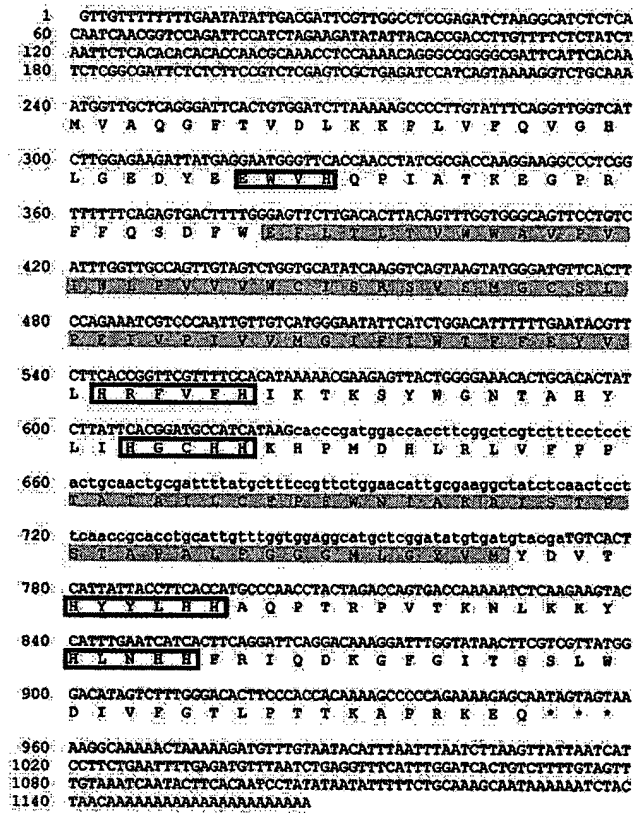


FIG. 4. **Nucleotide and amino acid sequence of the *A. thaliana* *FAH1* ORF.** Marked are the histidine motifs (*boxed*), and the transmembrane domains (*shaded*). *Lowercase* nucleotide sequence indicates data not covered by any ESTs.

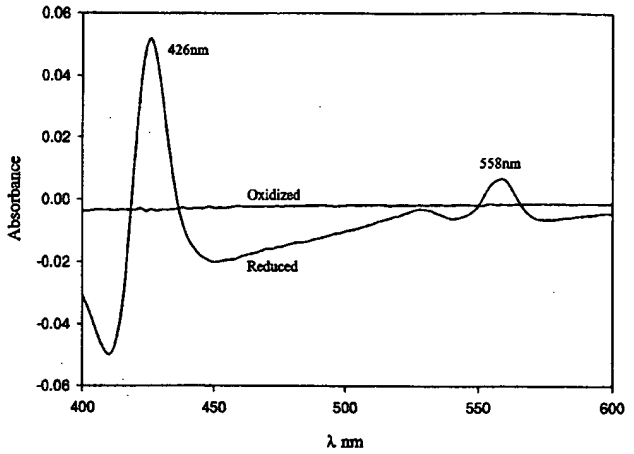


FIG. 5. **Oxidized *versus* reduced spectra of the recombinant cytochrome b_5 fragment.** *E. coli* (BL21(DE3) pLysS) containing the pAM154 plasmid were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 20 h at 30 °C. After cell lysis and centrifugation, air-oxidized and sodium dithionite-reduced absorbance spectra were recorded for the soluble protein extract.

predictions[4] for YMR272C and its homologs show the two hydrophobic domains, each of which is sufficient for two passes of the membrane bilayer (Fig. 6). As expected, there is a striking conservation of size and hydrophobicity of these transmembrane domains among the four Fah1p homologs. These data, taken together with the presence of the histidine motifs, suggest a similar topology to that proposed for *OLE1*. The relative positioning of the histidine motifs to the transmembrane domains are also consistent with the findings of Shanklin and co-workers that these are usually located close to, but not within, the membrane-spanning sections (19, 39) (Figs. 2 and 4). These properties suggest that the active site is assembled from the histidine-containing motifs on or near the membrane surface.

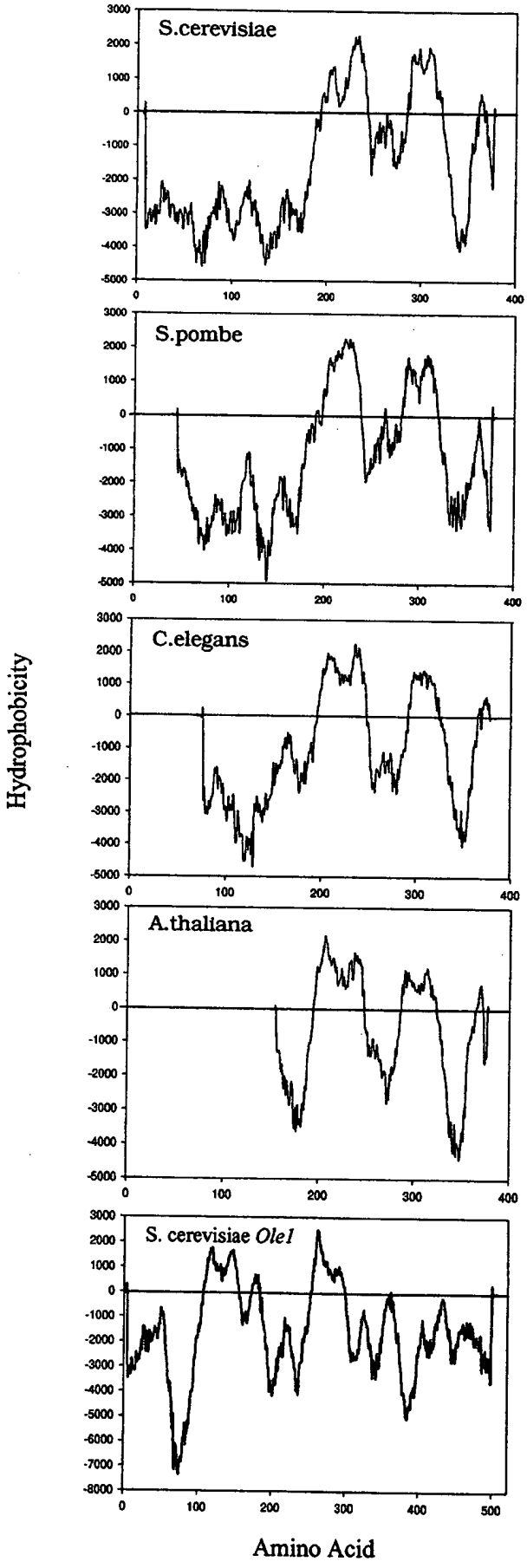
Northern Blot Analysis—A Northern blot of wild type DTY-11A RNA was probed with *OLE1*, cytochrome b_5, and the YMR272C sequences and quantified by phosphor imaging. The relative expression levels were calculated using the *S. cerevisiae* actin gene as an internal standard (Fig. 7). This blot shows that *OLE1* is expressed at a much higher level (~150-fold) when compared with YMR272C. The monounsaturated fatty acid products of the Ole1p represent approximately 60–70% species total cellular fatty acid content of yeast. By comparison, the relatively low level of YMR272C mRNA may indicate that it plays a minor role in lipogenesis.

Identification of an α-Hydroxylase Function—Given the similarities of YMR272C to *OLE1*, we examined the fatty acid compositions of wild type and YMR272C-disrupted cells. Analysis of midlogarithmic phase cells showed no observable difference in content or distribution of the long chain saturated and unsaturated 14–18-carbon fatty acid species. Those are predominantly found in membrane phospholipids and represent 94–96% of the total cellular fatty acids. However, significant differences in the fatty acid profiles of the less abundant very long chain fatty acid species were observed in the *fah1* Δ strain. Fig. 8 shows an alignment of the fatty acid methyl ester GC profiles from wild type (DTY-11A) and *fah1* Δ strains. Analysis of these very long chain fatty acids revealed that the disruption strain had an apparent increase in a peak at a 17.5-min retention time and a much reduced peak with a 38.5-min retention time. These two peaks were subsequently identified by mass spectroscopy as the 26:0 and the α-HO 26:0 fatty acid methyl esters. Their identification was further confirmed by the GC retention times and mass spectra of authentic standards (data not shown).

Fatty Acid Analysis of Sphingolipids—Very long chain fatty acids (C20–C28) are synthesized in yeast by elongation systems that act independently of the fatty acid synthase complex (4). These longer chain species are a minor component, representing approximately 4%, of the total cellular fatty acids. In wild type cells, the 26-carbon species is the most abundant very long chain acid, and it is almost exclusively found in sphingolipids. The hydrophobic ceramide portion of these lipids is composed of the long chain base phytosphingosine, which is amide-linked to a very long chain fatty acid, the majority of which is hydroxylated at the α-position (5, 6). It is suggested that the reaction that forms those species involves the direct hydroxylation of a sphingolipid bound fatty acid (8, 9) and that this process requires a cytochrome b_5 as the intermediate electron donor (7).

To demonstrate that *fah1*Δ cells are defective in the α-hydroxylation of sphingolipid-bound 26:0, the fatty acyl composition of sphingolipids was compared with that of the glycerolipid fraction and total cellular fatty acids. The *N*-acyl bond that links fatty acids to the long chain base to form ceramide is stable to mild alkaline hydrolysis, whereas the *O*-acyl bond found in phospholipids and glycerides is alkali-labile. To determine the sphingolipid fatty acyl content, 5% trichloroacetic acid-washed cells of DTY-11A and the *fah1* Δ strains were first subjected to alkaline hydrolysis to release glycerolipid fatty acids. The remaining lipids from the cell pellet were then extracted as described by Pinto *et al.* (30), a procedure used to quantitatively solubilize sphingolipids. The sphingolipid frac-

[4] Available on the World Wide Web at http://ulrec3.unil.ch/software/TMPRED_form.html.



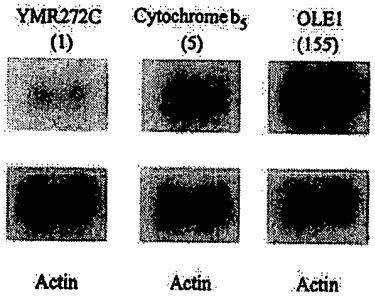


FIG. 7. **Northern blot analysis.** A Northern blot of DTY-11A wild type RNA was hybridized with ^{32}P-labeled probes to *OLE1*, cytochrome b_5, and YMR272C (*FAH1*). The relative expression levels were calculated using the *S. cerevisiae* actin gene as an internal standard.

tion extracted by that procedure was then subjected to acid methanolysis to determine the acyl content of those lipids. Fig. 9, *a* and *b*, compares the GC profiles of total fatty acid methyl esters of trichloroacetic acid-washed wild type DTY-11A and the *fah1* Δ cells. As previously shown (Fig. 8), the gene-disrupted strain shows the loss of the α-HO 26:0 peak and an increase in the 26:0 peak. Fig. 9, *c–f*, shows fatty acid methyl esters derived from the extraction procedure. *Traces c* and *d* are derived from the sphingolipid fraction. These fractions contain very low levels of 14–18-carbon species, which is typical of sphingolipids. As expected, Fig. 9*c* (wild type) shows that the very long chain fatty acids are predominantly the 26-carbon species of which ~80% is hydroxylated. Fig. 9*d*, however, shows that the primary sphingolipid species in *fah1*Δ is 26:0 and that the α-HO 26:0 peak represents less that 0.5% of that fraction. The residual HO 26:0 appears to be an intermediate product of the very long chain fatty acid elongation cycle rather than a product of an independent hydroxylase.

Fig. 9, *e* and *f*, shows fatty acid methyl esters from the alkali-labile lipids removed in the extraction procedure. The low amount of α-HO 26:0 in the wild type alkali-labile fraction in *trace e* (~0.1%) demonstrates, first, that virtually all sphingolipids are retained in the cell pellet by this method and, second, that there is a pool of 26:0 fatty acid that is not sphingolipid-bound. This is consistent with earlier reports that some 26:0 species are found in triglycerides (6). The absence of α-HO 26:0 in this pool also demonstrates the specificity of the hydroxylating enzyme for sphingolipid acyl groups. This observation is further supported by reports of mutant strains of *S. cerevisiae* that do not make sphingolipids when cultured without the sphingolipid long chain base phytosphingosine (9). These mutants are auxotrophic for long chain bases, and when grown with phytosphingosine they make sphingolipids in normal amounts. Suppressor mutants of these strains bypass the need to synthesize this long chain base by making novel inositol glycerophospholipids that structurally mimic sphingolipids and therefore compensate for some sphingolipid function(s) necessary for growth. These phosphatidyl inositol-containing lipids contain one molecule of 26:0 fatty acid (5) but apparently do not contain hydroxylated 26:0, suggesting that these *O*-acyl glycerolipids are not substrates for hydroxylation.

GAL1 Expression of the S. cerevisiae and A. thaliana ORFs—To demonstrate that the PCR-cloned genes were able to repair the disruption strain phenotype, the YCpGAL, pAM132, and pAM133 plasmids were independently transformed into the *fah1*Δ strain. Cells were grown in galactose-containing

Fig. 6. **Transmembrane prediction.** The TMpredict program (39) was used to analyze the homologous Fah1p proteins and Ole1p sequences.

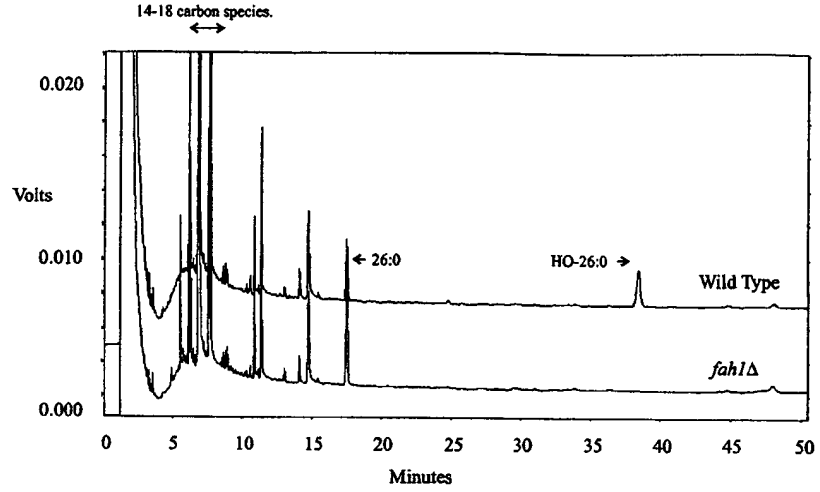


FIG. 8. **GC analysis of fatty acid methyl esters of wild type and *fah1Δ* strains.** Fatty acid methyl esters were prepared and analyzed by gas chromatography as described under "Materials and Methods." Under those conditions, 14–18-carbon fatty acid methyl esters have retention times of between 6 and 8 min. The 26:0 and hydroxy-26:0 species have retention times of 17.5 and 38.5 min, respectively.

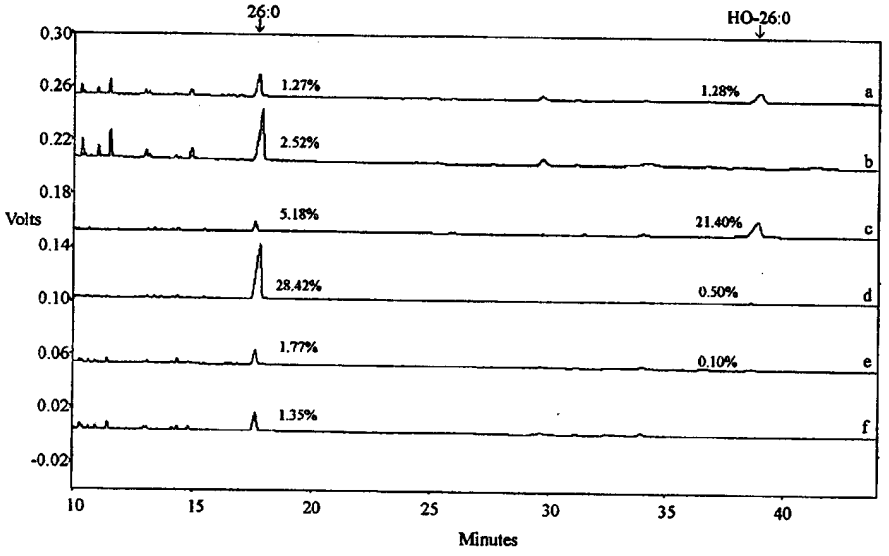


FIG. 9. **fah1Δ cells are defective in the α-hydroxylation of sphingolipid-bound 26:0 fatty acid.** The fatty acyl composition of sphingolipids was compared with that of the glycerolipid fraction and total cellular fatty acids. Percentages indicated refer to the wild type percentage of total fatty acids. Total cellular fatty acids were extracted from trichloroacetic acid-washed wild type DTY-11A (*a*) and YMR272C (*fah1Δ*)-disrupted (*b*) cells by HCl methanolysis. Sphingolipids were extracted from whole cells as described by Pinto *et al.* (30). After drying under nitrogen, these were then subjected to HCl methanolysis. The sphingolipid-associated fatty acids are shown for wild type DTY-11a (*c*) and the *fah1Δ* (*d*) strain. The alkaline-labile fraction (see "Materials and Methods") was also dried and further extracted by HCl methanolysis. Non-sphingolipid-associated fatty acids are similarly shown for wild type DTY-11A (*e*) and the *fah1Δ* strain (*f*).

media, sphingolipids were extracted, and fatty acid methyl esters were prepared as described under "Materials and Methods."

Analysis of sphingolipid-derived very long chain fatty acids revealed an approximately 40-fold reduction of α-HO 26:0 and a complementary increase in 26:0 in the gene-disrupted *fah1Δ* strain as compared with wild type (Fig. 10). Expression of the *S. cerevisiae FAH1* gene from the *GAL1* promoter (pAM132), restored the α-HO 26:0 fatty acid to wild type levels in the *fah1Δ* strain. Expression of the *A. thaliana* gene (pAM133), which does not contain the cytochrome b_5 domain, in the *fah1Δ* strain produced an approximately 25-fold increase in α-HO 26:0 and reduced levels of its 26-carbon precursor, indicating that it plays a similar role in very long chain fatty acid hydroxylation, apparently using an alternate electron donor. This activity of the *A. thaliana* homolog, which lacks a cytochrome b_5 domain, in the *fah1Δ* strain, parallels the observation of Stukey *et al.* (38) that the rat Δ-9 desaturase, which also lacks a cytochrome b_5 domain, functions in an *ole1Δ* strain.

Conclusions—We believe this to be the first report of the identification of a gene that acts in the hydroxylation of very long chain fatty acids. Extraction of sphingolipids demonstrates the specificity of this hydroxylase activity for the very long chain fatty acids associated with these lipids. Presumably, it encodes for the hydroxylating enzyme or one of its components, although conclusive proof will require expression and

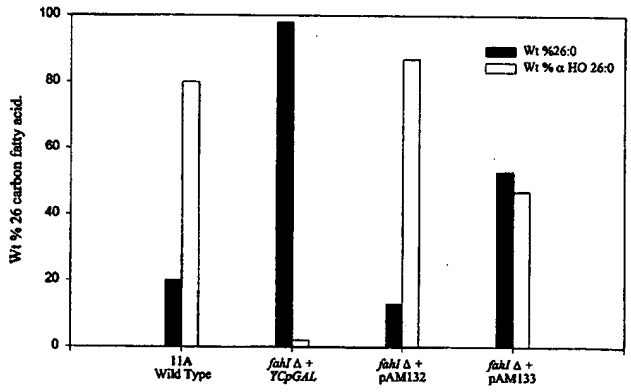


FIG. 10. **GAL1 expression of the *FAH1* gene.** Sphingolipids were extracted and fatty methyl esters derived from galactose-grown cells of wild type DTY-11A and the *fah1Δ* strain carrying the YCpGAL, pAM132, and pAM133 plasmids. The percentages refer to wild type percentage of 26 carbon fatty acid.

demonstration of its activity in a heterologous system. The ability of the homologous *A. thaliana* gene to substantially repair the hydroxylation defect also reinforces the idea that the gene encodes a component of the hydroxylation system.

As would be expected from the similarity of the reaction mechanisms for desaturases and hydroxylases (19), this *S.*

cerevisiae α-hydroxylase gene, *FAH1*, has a similar topology to *OLE1*. Both possess two hydrophobic domains, each capable of spanning the membrane twice, and contain the histidine-rich motifs associated with coordination of the μ-oxo-bridged diiron cluster. Surprisingly, both *S. cerevisiae* genes (*OLE1* and *FAH1*) have fused cytochrome b_5 domains. Because the positioning of these b_5 domains differ, it would appear that the two fusions occurred as independent evolutionary events rather than the divergence of a common b_5 fused ancestral gene. The closer homology of the b_5 domain of *FAH1* to the native cytochrome b_5 may reflect a more recent evolutionary fusion event than that which selected for the b_5 fusion to the Δ-9 fatty acid desaturase. It is still not clear why *S. cerevisiae* has evolved these two cytochrome b_5 fusion proteins, but one can speculate that their presence confers some evolutionary selectable advantage.

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